PRODUCTION OF BIOETHANOL FROM TAPIOCA STARCH USING *Saccharomyces serevisiae*: EFFECT OF INOCULUM CONCENTRATION AND TEMPERATURE

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I declare that this thesis entitled “Production of Bioethanol from Tapioca Starch Using *Saccharomyces cerevisiae*: Effect of Inoculum Concentration and Temperature” is the result of my own research except as cited in references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.”

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Special Dedication to my family members,
my friends, my fellow colleague
and all faculty members

For all your care, support and believe in me.

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ABSTRACT

The inoculum concentration of *Saccharomyces cerevisiae* yeast and the temperature on ethanol production were studied. The optimum conditions of starch hydrolysis such as substrate and enzymes concentration, pH and the time required for the enzymatic action were fixed during the entire process study. The two-step enzymatic hydrolysis of tapioca by commercially available α-amylase and glucoamylase were employed. The fermentation process was run in 250 mL shake flasks. Based on the results, the highest yield of ethanol was 20.7 wt% which was produced at 36°C for the effect of temperature study. However, for inoculum concentration study, at 20% (v/v), the yield of ethanol was 21 wt% which was the highest.

ABSTRAK

Kepekatan inokulum bagi yis *Saccharomyces cerevisiae* dan suhu bagi proses proses penghasilan etanol telah dikaji. Keaadaan optimum bagi hidrolisis kanji seperti kepekatan substrat dan enzim, pH dan masa yang diambil untuk tindakan enzim telah digunakan dalam proses kajian ini. Selain itu, dua langkah bagi proses hidrolisis enzim oleh α-amylase dan glucoamylase telah digunakan. Proses fermentasi dijalankan di dalam kelalang 250 mL. Berdasarkan data yang diperolehi, kadar etanol yang tertinggi adalah 20.7 wt% yang dihasilkan pada suhu 37°C bagi kajian kesan suhu. Bagaimanapun, untuk kajian kesan kepekatan inokulum, pada nilai kepekatan inokulum 20% (v/v), kadar etanol yang terhasil ialah 21 wt% di mana adalah nilai yang tertinggi.

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LIST OF SYMBOLS/ABBREVIATIONS

Ca^{2+}	-	ion calcium
CO_2	-	carbon dioxide
DE	-	dextrose equivalent
DNS	-	Di-Nitro Salicylic Acid
DP	-	degree of polymerization
EU	-	European Union
Glu.	-	Glucose
g	-	gram
h	-	hour
KNU	-	kilo
LODP	-	leveling off degree of polymerization
mg/L	-	milligram per liter
Mg^{2+}	-	ion magnesium
Min	-	minutes
mL	-	mililiter
Mm	-	megameter
OPEC	-	Organization of Petroleum Exporting Country
v/v	-	volume per volume
v/w	-	volume per weight
w/v	-	weight per volume
w/w	-	weight per weight
µg/mL	-	microgram per mililiter
%	-	percentage
°C	-	degree Celsius
°F	-	degree Fahrenheit
µmol	-	micromole

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Ethanol or ethyl alcohol (CH_3CH_2OH) is one of the most versatile oxygen-containing organic chemicals. Ethanol act as a solvent, a germicide, a beverage, an antifreeze, a fuel, a depressant and as a chemical intermediate for other organic chemical. The use of fermentation-derived ethanol or so called bioethanol as an automotive fuel additive to enhance octane and reduce emissions has seen an explosive growth over the last 12 years worldwide (Logsdon, 1994).

World bioethanol production in 2003 was 23 Mm^3 (Berg, 2004). The major world producers are Brazil and United States, which together account for about 80% of the world production. Agricultural raw materials rich in fermentable carbohydrates could be converted to yield the fermentable sugars. Main feedstock for bioethanol production is sugarcane (in Brazil) and corn grain (USA) (Mojović *et al*., 2006).

Basically, major carbohydrate-containing substrates such as wheat, rice and starch are commonly used sources of food for a major population in Malaysia. Therefore, starchy substrate such as tapioca could be exploited for ethanol production. Recently, Aggarwal *et al.* (2001) estimated that the starch content of tapioca powder was estimated to be 95% and moisture content 2%. Moreover, owing to its high carbohydrate content, tapioca provides one of the most efficient sources of starch. This raw material has not yet been fully exploited in highly technical industrial enterprises for ethanol production. Since the use of starch-based raw

materials for ethanol production is not a common practice in Malaysia, it is imperative to optimize the conditions for the economical hydrolysis of the starchy substrate to produce sugars for subsequent fermentation. Starch is a reserve polysaccharide of plant origin, which cannot be converted to sugars easily. Starch saccharification requires prior gelatinization by heat treatment, liquefaction by a-amylase and conversion to sugars by glucoamylase (Aggarwal *et al*., 2001). There are two major processes on converting from rich fermentable carbohydrates materials to ethanol which are enzymatic hydrolysis (from carbohydrates to sugars) and fermentation by microorganisms such (from sugars to ethanol).

1.2 Objective

The aim of this study/research is to determine the optimum conditions of fermentation process for the production of bioethanol from tapioca starch. Hence, the objectives of this research are:

i. To study the effect of inoculum concentration on the production of bioethanol from tapioca starch
ii. To study the effect of temperature on the production of bioethanol from tapioca starch.

1.3 Scope of Study

Bioethanol production will conducted by two-step process; enzymatic hydrolysis followed by fermentation process. The scope for this study is to determine the yield of bioethanol that can be produced from locally available tapioca starch during fermentation process. Various inoculums concentration of *Saccharomyces cerevisiae* yeast and temperature will be investigated. The optimized conditions of starch hydrolysis from literature survey such as substrate and enzymes concentration, pH and the time required for the enzymatic action will be employed during the process study.

1.4 Problems Statement

Due to the diminishing fossil fuel reserves, alternative energy sources need to be renewable, sustainable, efficient, cost-effective, convenient and safe (Chum and Overend, 2001). In the past decades, the production of ethanol has been focused and considered as an alternative fuel for future since fossil fuel is currently depleted (Najafpour *et al*., 2003). Furthermore, the use of ethanol from renewable lingocelluloses resources may improve energy availability, decrease air pollution and diminish CO_2 accumulation. Ethanol is found to be biodegradable, low in toxicity and cause little environment pollution (Azrul, 2006).

Since ethanol has become one of the major sources as an alternative fuel, it is important to investigate on the production of ethanol as that the process will be time-reducing and cost-effective. Earlier developments in the conversion of starch to ethanol involved acid hydrolysis. However the production of by products such as furfural and formic acid has resulted in lower yields of alcohol and inhibited yeast growth. Acid-hydrolysis also caused the degradation of sugars to toxic 5-hydroxymethylfurfural resulted in the undesirable off-flavours. Moreover, it is not possible to achieve dextrose equivalent (DE) greater than 55 without generation off-taste. Therefore, most acid-hydrolysis has been replaced by enzymatic hydrolysis (Aggarwal *et al*., 2001).

Basically, most of the raw materials used for the production of bioethanol were corn grain and sugar cane (Mojović *et al*., 2006). However, it is also important to see the potential of the other agricultural raw materials rich in fermentable carbohydrates such as tapioca since it is available in Malaysia and cheaper compare to the others.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

The demand for ethanol is increasing in recent years because of its wide use in chemical, potable and motor-fuel industries (Suresh *et al*., 1999). Research and development efforts directed toward commercial production of ethanol as the most promising biofuel from renewable resources have increased (Mojović *et al*., 2006). In many developed countries in Europe and in USA, the use of bioethanol as an alternative fuel or a gasoline supplement in the amounts up to 15% is highly recommended or even required as an ecologically favorable fuel oxygenates (Burnes *et al.,* 2005). Concerning the European Union (EU), a new directive was accepted in November 2001 that requires of members states to establish legislation about utilization of fuels from renewable resources. In 2005, this utilization should cover 2% of the total fuel consumption. This quota is expected to increase to 5.75% in 2010 and furthermore. Some member states like Finland, Sweden or Austria have already fulfilled this quota (Berna, 1998).

Utilization of starch and cellulose substrates for ethanol production is now preferred for economic reasons (Suresh *et al.*, 1999). An important issue regarding the bioethanol production is weather the process is economical. Research efforts are focused to design and improve a process, which would produce a sustainable transportation fuel. A low cost of feedstock is a very important factor in establishing a cost effective technology (Mojović *et al*., 2006).

2.2 Background of Ethanol

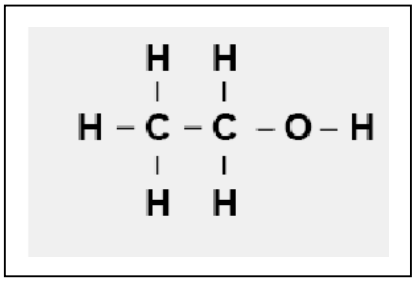

Figure 2.1: Ethanol Structure

Ethanol (Ethyl Alcohol or Grain Alcohol, C_2H_5OH) can be defined as clear, colorless flammable oxygenated hydrocarbon with boiling point 173.5°F in the anhydrous (Figure 2.2) (Azrul, 2006). Ethyl alcohol is well known as a constituent of alcoholic beverages. As a beverage ethanol had been prepared and used long ago by the Egyptian pharaohs. Some indication of the antiquity of the knowledge of ethyl alcohol is the fact that Noah is believed to have built a vineyard in which he grew grapes that he fermented into a type of alcoholic beverage (Logsdon, 1994).

2.3 Production of Ethanol

Industrial ethyl alcohol can be produced synthetically from ethylene or by the fermentation of sugar, starch or cellulose.

2.3.1 Synthetic Process

There are two main processes in the synthesis of ethyl alcohol from ethylene. The earliest to be developed was the indirect hydration process, variously called the strong sulfuric acid-ethylene process, the ethyl sulfate process, the esterification-hydrolysis process, or the sulfation-hydrolysis process. This process is still use in Russia. The other synthesis process, designed to eliminate the use of sulfuric acid

and which, since the early 1970's, has completely supplanted the old sulfuric acid process in the United States, is the direct hydration process. This process involves the catalytic vapor-phase hydration of ethylene. There are other synthetic methods that have been investigated but have not become commercial. These include, for example, the hydration of ethylene in the presence of dilute acids (weak sulfuric acid process); the conversion of acetylene to acetaldehyde, followed by hydrogenation of the aldehyde to ethyl alcohol (Logsdon, 1994).

2.3.2 Fermentation Process

Fermentation is one of the oldest chemical processes known to man. It is used to make a variety of products, including fuel, foods, flavorings, beverages, pharmaceuticals and chemicals. At present, however many of the simpler products such as ethanol are synthesized from petroleum feedstocks at lower cost. Ethanol production by fermentation, excluding that for beverages, had been declining in United States since synthetic ethanol was introduced in the 1930s, because of the low cost and assured availability of ethylene. The quadrupling of the selling price of crude petroleum by the Organization of Petroleum Exporting Countries (OPEC) in 1973 had a profound impact on fermentation processes for producing ethanol. Furthermore, the unstable price and the availability of crude petroleum had caused the fermentation become an alternatives process to produce ethanol (Logsdon, 1994). Recently, Baras *et al*. (2002) had reported around 60% of the ethanol is produced by fermentation the major world producers are Brazil and the US, which together account for about 80% of the world production.

2.4 Raw Materials for Ethanol Fermentation

Ethanol can be derived by fermentation processes from any material that contains sugar or compounds that can be converted to sugar. The many and varied raw materials used in the manufacture of ethanol via fermentation are conveniently classified under these three types of agricultural raw materials: sugar, starches and cellulose materials. Sugar (from sugar cane, sugar beets, molasses, or fruit) can be converted to ethanol directly. Starches (from grains, root crops) must first be hydrolyzed to fermentable sugars by the action of enzymes from malt or molds. Cellulose (from wood, agricultural residues or waste sulfite liquor from pulp and paper mills) must likewise be converted to sugars, generally by the action of mineral acids. Once simple sugars are formed, enzymes from yeast can readily ferment them to ethanol (Logsdon, 1994).

2.4.1 Sugars

The direct fermentation of sugar cane juice, sugar beet juice, beet molasses (by-product in the production of beet sugar), fresh and dried fruits, cane sorghum, whey and skim milk had been considered as a means of obtaining ethanol. However, none of these raw materials could compete economically with molasses. Although the manufacture of ethanol from the sugar- containing waste products of the fruit industry appears to be a highly desirable operation, particularly as a means of reducing stream pollution in the vicinity of canning plants, such production is costly because of the need to remove most of the water (as much as 97%) contained in the waste product (Logsdon, 1994).

2.4.2 Starches

Starch occurs naturally in most plant tissues, including roots and tubers, cereal grains, vegetables and fruits. The principal components of starch are amylase and/or amylopectin. Amylose is an essentially linear polysaccharide composed of (1-4)-linked α-D-glucopyranosyl units. Because of its helical structure, amylase is able to complex with hydrophobic molecules. Complexed amylase molecules retrograde less effectively. Hence, molecules complexed with hydrocarbon chains provide greater stability to foods. Amylopectin has a branch-on-branch structure. Amylopectin molecules are composed of chains of (1-4)-linked α-D-glucopyranosyl units; branches are formed by joining these chains with α-D-(1-6) linkages. The average chain length is 20 to 30 units, although branch points are not equally spaced (BeMiller, 2000).

Fermentation of starch from grain is somewhat more complex than fermentation of sugars because starch must first be converted to sugars and then to ethanol. Starch is converted enzymatically to glucose either diastase present in sprouting grain or by fungal amylase. The resulting dextrose is fermented to ethanol with the aid of yeast, producing CO_2 as a coproduct. Other by-product depends on the type of process (Logsdon, 1994).

2.4.3 Cellulosic Materials

The technology for converting the cellulosic materials into ethanol is available, but the stoichiometry of the process is disadvantageous. Even if each step in the process of the conversion of cellulose to ethanol proceeds with 100% yield, almost two-thirds of the mass would disappear during the sequence, most of it as carbon dioxide in the fermentation of glucose to ethanol. This amount of carbon dioxide leads to a disposal problem rather than to a raw material credit.

Starch and cellulose are both polymers of glucose, but cellulose is much more difficult to hydrolyze to the sugar. Its structure is more crystalline which protects the internal bonds from hydrolysis, and cellulose in plants is protected by lignin, a polyphenolic material that forms a seal around the cellulose for further protection against hydrolysis. Cellulosic wastes also contain substantial amounts of hemicellulose, which is a polymer of pentoses. The aqueous mineral acids used to hydrolyze the cellulose to glucose destroy much of the sugars, particularly the pentoses, in the process (Logsdon, 1994).

2.5 Acid Hydrolysis

Initially, acid hydrolysis appears to be a relatively efficient means of accessing and breaking down cellulose. The hydrogen ion, therefore, does not face the problem of accessibility compared to cellulase enzymes. Furthermore, the basic mechanism of the 20 hydrolysis of glycosidic bonds is relatively simple the mechanism is similar to the hydrolysis of other glycosides such as starch (α1-4 linked glucose chains, with α1-6 branches). Step 3 is the rate-limiting step of the process because of the formation of the high energy half-chair configuration by the cyclic carbonium ion. Initial hydrolysis rates are typically very rapid performed experiments to show that in the initial stages of the hydrolysis reaction, larger pore volumes do correspond to faster reaction rates. However, after limited hydrolysis, the reaction rate slows down considerably. The glycosidic bonds most susceptible to hydrolysis are those either at the surfaces or in the amorphous regions of cellulose. Rapid hydrolysis rates reflect hydrolysis activity in these regions and can be seen as a decrease in the degree of polymerization (DP) from several thousand to about 200. This point is referred to as the leveling off degree of polymerization (LODP). Further hydrolysis is much more difficult beyond the LODP because of the high crystallinity of the remaining cellulose molecules (Azrul, 2006).

This technique is practiced on commercial scale for glucose production from cellulose. The operation is carried out at an elevated temperature and glucose production efficiency by this process goes up to 50%. However, yield reductions

inherent in glucose degradation during dilute acid hydrolysis at high temperatures are not present in concentrated acid hydrolysis at lower temperature. This process depends on disruption of the crystalline structure of the cellulose by solution or swelling in the acid. The cellulose can then be rapidly hydrolyzed at low temperature to avoid degradation, making almost quantitative yields of glucose attainable. However, in the process, high capital cost is unavoidable because of expensive corrosion resistant equipment, acid recovery plants and higher operation costs (Ajit and Basant, 2003). Moreover, one of the major problems with hydrolyzates produced by acid hydrolysis is the poor fermentability caused by the presence of inhibitors in the hydrolyzates. Furfural is known to be one of the most important of these inhibitors. It is a breakdown product from pentoses and is formed in a browning reaction during hydrolysis in the presence of strong acids. It therefore may be impossible to completely avoid furfural formation in a chemical hydrolysis process designed to give a high sugar yield (Taherzadeh *et al*., 1999).

2.6 Enzymatic Hydrolysis

Lately interest in the enzymatic hydrolysis to get glucose and ethanol has increased as this involved milder condition. Although enzymatic hydrolysis of starchy substrates can give 100% yield of glucose, the reaction is much slower as compared to acid hydrolysis. But the severe problem (corrosion of reactor) during acid hydrolysis process can be avoided by enzymatic hydrolysis process (Ajit and Basant, 2003). Basically, there are two major processes involve in enzymatic hydrolysis; liquefaction and saccharification. The main role of enzymatic hydrolysis is to effectively provide the conversion of two major starch polymer components (amylose and amylopectin) to fermentable sugar that could subsequently be converted to ethanol by yeast.

2.6.1 Liquefaction

The starch content of tapioca powder was estimated to be 95% and moisture content 2%. Liquefaction under pressurized steam was found to be more effective than that of using water bath at since the slurry of tapioca powder was liquefied in a significantly shorter time. The slurry of 35% consistency, which could not be liquefied within 4 hours under either temperature conditions, was discarded keeping in view the time as one of the important considerations in liquefaction process. A slurry of 25% consistency was found to be more appropriate for this process as the liquefaction took only 45 min at 104°C and 120 min at 95°C. Since the pressurized heat yielded better results, the liquefaction in further experiments was carried out under this condition, keeping the total time requirement of 45 min using 25% slurry of tapioca powder. The shorter period of liquefaction in an autoclave could be due to the uniform heating under pressure and constant maintenance of temperature throughout (Aggarwal *et al*., 2001).

The liquefaction was achieved within 45 min as visualized by starch–iodine reaction. This liquefaction protocol provided additional advantages as the gelatinization and liquefaction steps were carried out in a single-step thus saving the energy incurred for the sterilization of starchy substrates. The optimized concentration of enzyme was found to be 0.15%, v/w. Liquefaction took twice as long on reducing the enzyme dose from 0.15 to 0.10% for 25% slurry. In order to determine the optimum pH for liquefaction, tapioca slurry was prepared in buffer of pH values 5.0, 5.5. 6.0, 6.5 and 7.0, and liquefied using 0.15% enzyme dose. The results of starch–iodine reaction showed that efficient liquefaction of tapioca was achieved in a pH range of 6.5–7.0. To examine the effect of divalent ions on the process of liquefaction, various concentrations of calcium chloride and magnesium sulphate providing Ca^{2+} and Mg^{2+} ranging from 25 to 250 mg/l were added to the slurry. The results obtained suggested a requirement of Ca^{2+} was required for liquefaction process of similar duration in presence of Ca^{2+}. Therefore to reduce the enzyme dose from 0.15 to 0.10% (w/v), the calcium chloride supplementation was optimized and found to be 120 mg/l of Ca^{2+}. At concentrations of calcium lower than

120 mg/l, liquefaction required 0.15% enzyme concentration i.e. 50% more. Magnesium sulphate did not show any effect on liquefaction (Aggarwal *et al*., 2001).

2.6.2 Saccharification

For this step, the concentrated crude preparation of glucoamylase from *Aspergillus sp*. was used. The maximum amount of sugars (up to 90%) was produced after 24 h. Glucose was the main sugar in the enzymatic hydrolysate of tapioca starch as detected by paper chromatography. Maximum saccharification occurred at 60°C, at higher temperature the rate of saccharification reduced substantially. The optimum pH for the saccharification was found to be 5.0. With above optimized conditions for the saccharification (time, temperature and pH), the concentration of glucoamylase was optimized (Aggarwal *et al*., 2001).

The saccharification improved with the increasing enzyme units within the range of 10–30 U/ml. To achieve 92% saccharification, the enzyme was needed at the concentration of 30 U/ml, which was close to the expected value. Higher units did not prove effective. Effect of addition of divalent ions on the process of saccharification was studied by the addition of calcium chloride, magnesium and zinc sulphate to provide these ions in the range of 25–250 mg/l. Results obtained for the level of saccharification in presence of these ions, indicated that irrespective of type and the concentration saccharification was similar in all cases as in control showing no effect of divalent ions. The parameters must be standardized for the type of substrate to be hydrolysed. It seems to be a common practice of producing hydrolysate from dilute slurries and to concentrate low-sugar hydrolysates, dilute slurries requires lesser time for saccharification. In many cases relatively high doses of glucoamylases and other maceration enzymes besides amylases such as xylanase, cellulase and pectinase are necessary to saccharify various starch-containing substrates efficiency. Moreover, the efficiency of an enzymatic starch saccharification process depends on the activity of the glucoamylase and also on the purity of enzyme (Aggarwal *et al*., 2001).